Hypoxia-Inducible Factor-1 α and -2 α Additively Promote Endothelial Vasculogenic Properties

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Key Words

Hypoxia-inducible factors • Endothelial cells • Cytokine expression • Vasculogenesis

Abstract

Objective: While both play a role in the transcriptional response of hypoxic endothelial cells (ECs), hypoxia-inducible factor-1 α (HIF-1 α) and HIF-2 α differ in their transactivation sites, pointing at potentially different target genes. We studied the discrete and common effects of HIF-1 α and HIF-2 α on the cytokine expression and vasculogenic properties of ECs. **Methods and Results:** H5V and bovine aortic ECs were transfected to express HIF-1 α , HIF-2 α or both. Overexpression of HIF-1 α or HIF-2 α and, to a greater extent, cotransfection of HIF-1 α and HIF-2 α resulted in EC activation, as revealed by analysis of the adhesion capacities and adhesion molecule surface expression of ECs. From the paracrine aspect, conditioned medium from HIF-expressing ECs was found to promote the migration and tube formation capacity of wild-type ECs, mostly following HIF-1 α and HIF-2 α co-expression. Antibody arrays revealed altered expression of multiple cytokines, pointing at consistent additive effects

of HIF-1 α and HIF-2 α on angiogenic protein expression. Finally, HIF-1 α and HIF-2 α additively promoted vessel formation in vivo, as demonstrated by a Matrigel angiogenesis assay. **Conclusion:** Our results further clarify the functional roles of HIF-1 α and HIF-2 α in ECs and for the first time demonstrate a common contribution of HIF-1 α and HIF-2 α to vasculogenesis.

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Introduction

Hypoxia constitutes a fundamental regulatory mechanism of angiogenesis during embryonic vascular development, as well as in various physiological and pathological conditions in the adult [1]. In endothelial cells (ECs), hypoxic conditions drive the transcription of multiple genes which control vascular function and remodeling [2]. Hypoxia-inducible factor (HIF) is the main regulator of the cellular response to low oxygen levels in mammals, playing a key role in controlling glycolysis, angiogenesis, erythropoiesis, vascular tone and cell survival [3]. HIF constitutes a heterodimer of an oxygen-de-

pendent α -subunit and a constitutively expressed β -subunit that interact through mutual basic helix-loop-helix (bHLH) domains. Under normoxia, the HIF α oxygen-dependent degradation (ODD) domain is hydroxylated by prolyl hydroxylases and driven to ubiquitination by the von Hippel-Lindau (VHL) protein [4]. In the absence of molecular oxygen, HIF α hydroxylation is abrogated, leading to its cellular accumulation. HIF subunits then heterodimerize, bind to hypoxia response elements (HREs) on target genes through the N-terminal basic DNA binding domain and induce transcriptional activity.

The 2 principal HIF α subunits, HIF-1 α , which is ubiquitously expressed, and HIF-2 α , which shows a spatially restricted expression [5], both play important roles in vascular development and endothelial function. Several genes encoding growth factors and tyrosine kinase receptors essential for EC growth and function (e.g. *Epo*, *Vegf*, *eNos* and *Flk-1*) were shown to comprise HIF-binding sites in their promoter/enhancer sequences [6–10]. Despite their high degree of homology, several studies have suggested nonoverlapping roles of HIF-1 α and HIF-2 α in hypoxic gene regulation [11–14]. HIF-1 α and HIF-1 β knockout mouse embryos showed extensive EC death, cardiovascular malformations and embryonic lethality [15, 16]. HIF-2 α embryonic ablation has been suggested to cause variable phenotypes, including death from vascular defects [17, 18], impaired catecholamine synthesis and bradycardia [19], neonatal lung immaturity [20], multiorgan pathology and biochemical disorders [21]. Recently, endothelial-specific HIF inactivation was associated with embryonic cardiovascular abnormalities and early lethality, mimicking the Tie-2-deficient phenotype [22]. In mature ECs, the transcriptional response of HIF-1 α to hypoxia regulates the expression of multiple genes involved in cell survival, growth and metabolism [2, 23]. Accordingly, loss of HIF-1 α interferes with hypoxia-induced vascular endothelial growth factor (VEGF) autocrine activation of ECs [24]. Compared to HIF-1 α , HIF-2 α preferentially activates EC transcription of different essential proangiogenic genes, through its binding to the HRE (e.g. *eNos* and *Vegf*) or other response elements (e.g. *Tie-2* and *Flk-1*) [5, 8–10]. Together, these data indicate that HIF-1 α and HIF-2 α might fulfill distinct and perhaps complementary roles in functional aspects of angiogenesis.

In the present study, we investigated the role of HIF-1 and HIF-2 in the expression of potential angiogenic target proteins in ECs, in correlation with their functional properties. Our findings may illuminate the HIF-mediated

endothelial response within the hypoxic environment, potentially contributing to future endothelium-targeted angiogenic gene therapy.

Materials and Methods

Animals and Cells

Male 8-week-old C57BL/6J mice were obtained from Harlan Laboratories, Jerusalem, Israel. H5V murine heart-derived ECs and bovine aortic endothelial (BAE) cells were grown at 37°C in 8% CO₂ in Dulbecco's modified Eagle's medium-F12 and Dulbecco's modified Eagle's medium-low glucose, respectively (Biological Industries, Beit Haemek, Israel), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FCS.

Plasmids and Antibodies

hHIF-1 α cDNA was cloned into the *NotI/BamHI* sites of p3XFLAG-myc-CMV-25 (Sigma-Aldrich) to yield Flag-HIF-1 α . hHIF-2 α cDNA cloned into the *KpnI/BamHI* sites of pcDNA3.1myc (Invitrogen) was kindly provided by Dr. F. Shibasaki, and pcDNA3.1 served as control vector. pCEP/HIF-1 α - Δ NB Δ AB was purchased from ATCC (MBA-7). Constructs were confirmed by DNA sequencing (Hylabs, Rehovot, Israel).

The following antibodies were used: mouse anti-Flag M2 monoclonal antibody (Sigma-Aldrich), mouse anti-c-myc 9E10 monoclonal antibody (Covance), anti-HIF-1 α antibody (R&D Systems), anti-HIF-2 α polyclonal antibody (Novus Biologicals), mouse anti- β -tubulin monoclonal antibodies (Sigma-Aldrich), fluorescein isothiocyanate (FITC) rat IgG2b isotype control, FITC antimouse CD106 (vascular cell adhesion molecule-1; VCAM-1), FITC antimouse CD54 (intercellular adhesion molecule-1; ICAM-1) (eBioscience) and rat anti-CD31 monoclonal antibody (BD).

Transfections

H5V and BAE cells were plated in 9-cm culture dishes and grown to 70% confluence. Six micrograms of empty vector, HIF-1, HIF-2 or HIF-1 + HIF-2 (3 + 3 µg) plasmid DNA were transfected into ECs using jetPEI™ reagent (Polyplus Transfection), according to the manufacturer's instructions. For cotransfections, HIF-1 or HIF-2 and HIF-dominant negative (HIFdn) plasmids were used (6 µg of each plasmid). In these assays, 12 µg of empty vector or dominant negative plasmid DNA served as control. Medium was changed 24 h after transfection and cells were grown for an additional 12–24 h before harvest.

Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from 3×10^6 cultured ECs by phenol-chloroform-isoamyl alcohol (Biological Industries). RNA was treated with DNase I (Ambion). RNA (2 µg/reaction) was transcribed using AMV reverse transcriptase (Promega). Amplification reactions were carried out using Red Taq Ready Mix (Sigma-Aldrich). Reaction conditions and cell cycle number were calibrated for each gene and primers to avoid saturation of the reactions (sequences available on request). Quantification was performed using Tina 2.0 software.

Western Blot Analysis

A total of 50 µg of whole-cell protein extract per sample, prepared from H5V and BAE cells, was separated on 8% polyacrylamide gel and electroblotted on nitrocellulose membranes. Flag-HIF-1α, myc-HIF-2α and β-tubulin were detected using the specific antibodies described above. Corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz), in combination with enhanced chemiluminescence reagent (Amersham), were used for signal detection.

Enzyme-Linked Immunosorbent Assay

The Human/Mouse Active HIF-1α DuoSet® Intracellular ELISA Development System (R&D) was used for the evaluation of HIF activity, according to the manufacturer's instructions. Briefly, a biotinylated double-stranded oligonucleotide containing the consensus HRE HIF binding site was incubated with H5V nuclear extracts. HIF-double-stranded oligonucleotide complexes were captured by immobilized antibodies specific for HIF-1α or HIF-2α. Unlabeled double-stranded competitor oligonucleotide was used to test the assay's specificity. Detection was performed utilizing streptavidin-horseradish peroxidase and an ELISA reader.

Adhesion Assays

For adhesion to fibronectin, 10⁴ H5V or BAE cells/well were seeded in 96-well plates coated with fibronectin for 30 min. For assessment of monocyte adhesion, ECs were seeded for 4 h until confluence, and 10⁵ U937 cells were then added to the wells for 30 min. Nonadherent cells were washed away, and adherent cells were stained using XTT-based colorimetry (Biological Industries). Optical density at 450 nm, proportional to viable cell number, was measured using an ELISA reader. Fibronectin-coated wells and EC-only seeded wells (standardized for each group) served as background, respectively.

Flow Cytometry Analysis

Forty-eight hours after transfection, H5V cells were gently removed from culture dishes using a cell scraper, washed and diluted in PBS containing 1% BSA. Cells were stained against mouse IgG₁ isotype control, ICAM-1 or VCAM-1 and analyzed by flow cytometry (FACScan; Becton Dickinson).

Migration Assay

H5V and BAE cells were grown 48 h after transfection in serum-free conditioned media. Media were then collected and loaded onto the lower chamber of a Boyden chamber (Neuroprobe) housing an 8-µm polycarbonate filter. Then, 5 × 10⁴ ECs in 50 µl of serum-free media were loaded into the top chamber. Migrating cells were fixed and stained with Diff-Quick Stain (Dade Behring AG) and counted in 4 high-power fields (×20).

Matrigel Tube Formation Assay

Twenty-four hours before the assays, Matrigel (BD) was stored at 4°C and transferred to ice immediately before coating/injection. H5V or BAE cells (2 × 10³ cells/well) were seeded in 96-well plates coated with Matrigel. Tube formation was evaluated after 8 and 16 h by phase-contrast microscopy (×40) and scored as described elsewhere [25]: 0, individual cells, well separated; 1, cells begin to migrate and align themselves; 2, capillary tubes visible,

no sprouting; 3, sprouting of new capillary tubes visible; 4, closed polygons begin to form, and 5, complex mesh-like structures develop.

Antibody Array

The RayBio® Mouse Cytokine Array C series 1,000 assay was performed according to the manufacturer's instructions. Briefly, 500 µg of whole-cell extract proteins were incubated with arrayed antibody membranes, which were exposed to a specific biotin-antibody cocktail. Signals were detected using labeled streptavidin and exposure on X-OMAT AR films. Signal quantification and data analysis were performed using Tina 2.0 software. The significance threshold was set as 1.5-fold, and proteins showing such an induction/repression in at least 1 experimental group are presented (means ± SD; p < 0.05).

In vivo Matrigel Angiogenesis Assay

Transfected H5V cells (2 × 10⁶) in 50 µl of PBS were mixed with 400 µl of liquid Matrigel and were subcutaneously injected into 4 groups of mice (n = 5). After 8 days, mice were sacrificed, and 5-µm-thick frozen sections were prepared from the excised plugs. Immunohistochemical assessment of vascular density was performed by CD31 and hematoxylin costaining. Quantitative analysis was performed under a light microscope (×20) in high-power fields (n = 10), employing Image J software (identifiable vessel number × mean vessel area). Additional sections were stained with DAPI, and optical fields (n = 5) were analyzed by fluorescence microscopy (×20 and ×40).

Statistical Analysis

Comparison between the groups was performed employing one-way ANOVA. The level of significance was set at p < 0.05. Results are expressed as means ± SD unless otherwise specified.

Results

Overexpression of HIFα Subunits in ECs

The plasmids used to modify HIFα expression in H5V and BAE cells included Flag-HIF-1α, myc-HIF-2α and a dominant negative (dn) form of HIF-1α (HIFdn), containing a deletion in the bHLH domain and lacking ODD and transactivation domains, thus competing with both endogenous HIFα forms [3, 22] (fig. 1a). First, human HIF-1α, HIF-2α or both were transfected into ECs (fig. 1b). Since the ubiquitination of HIFα subunits is saturable [14, 26, 27], HIFα protein stabilization under normoxia was achieved following transfection of a relatively high dose of expression vector (6 µg for HIF-1α or HIF-2α and 3 + 3 µg for cotransfection of HIF-1α + HIF-2α per 9-cm dish; fig. 1c). Then, the induction of the well-established HIFα common targets, VEGF and VEGF receptor (VEGFR)-2, was validated [5, 7, 9, 24] (fig. 1d).

Fig. 1. a The plasmids used to modify HIF α expression in H5V and BAE cells: Flag-HIF-1 α , myc-HIF-2 α and HIFdn, containing a deletion in the bHLH domain and lacking ODD and transactivation domains. PAS = Per/Arnt/Sim; NTAD = N-terminal transactivation domains; CTAD = C-terminal transactivation domains. **b** Presented are the representative results achieved by H5V transfections. Human (h) and mouse (m) HIF-1 α (6 μ g), HIF-2 α (6 μ g) or both (3 + 3 μ g) were transfected into ECs. Expression was evaluated by RT-PCR using GA3PDH as housekeeping gene. **c** At the protein level, the stabilization of HIF α subunits following transfection of an increasing dose of DNA plasmid was evaluated by an ELISA-based transcription factor assay, testing the cellular protein's ability to bind a consensus HRE. **d** The induction of HIF α common targets, VEGF and VEGFR-2, was assessed by RT-PCR. RNA data were quantified by Tina 2.0 software, standardized to GA3PDH and the control group. * $p < 0.05$; ** $p < 0.005$.

Cotransfection of HIFdn plasmid (6 μ g) and HIF-1 α or HIF-2 α (6 μ g) did not diminish HIF-1 α or HIF-2 α overexpression as evaluated by Western blot (fig. 1e). No change in the HRE binding signal was achieved by cotransfection of HIFdn and empty vector, implying no sig-

nificant basal expression of HIF-1 α or HIF-2 α in normoxia, as previously reported [14, 24, 26] (fig. 1f). However, total binding capacity in both HIF-1 α - and HIF-2 α -transfected ECs was significantly reduced in the presence of HIFdn.

Fig. 1. e Cotransfection of HIFdn plasmid (6 μ g) with each cell group was performed in order to attenuate HIF α effects. Immunoblot assay using α -Flag and α -myc antibodies confirmed HIF overexpression and that HIFdn did not diminish total HIF-1 α or

HIF-2 α overexpression. β -tubulin served as endogenous control. cont-nt = control-nontransfected. **f** Finally, HIF-1 α and HIF-2 α HRE-binding capacity in each cell group was assessed using hypoxic cells as positive control. ND = Not done.

HIF-1 α and HIF-2 α Commonly Promote the Adhesive Properties of ECs

Increased EC adhesion capacity plays a crucial role in EC growth and activation, particularly during new vessel sprouting [28]. We thus assessed the ability of two EC lines, H5V and BAE cells, to adhere both to fibronectin and monocytes following overexpression of HIF α subunits. In the fibronectin adhesion assay (fig. 2a), an increased number of adherent cells was observed in the HIF-1 α -transfected group as compared to control (1.77 ± 0.17 -fold, $p < 0.005$, and 1.57 ± 0.29 -fold, $p < 0.05$, in H5V and BAE cells, respectively), as well as in the HIF-2 α -transfected group (1.91 ± 0.18 -fold and 2.34 ± 0.09 -fold in H5V and BAE cells, respectively; $p < 0.005$ for both). Statistical significance was found between the HIF-1 and HIF-2 groups for all assays, excepting the H5V fibronectin assay, which showed a more prominent effect of HIF-2 α on adhesion capacity. Coexpression of HIF-1 α and HIF-2 α was associated with an increased EC adhesion capacity (2.6 ± 0.23 -fold and 2.54 ± 0.14 -fold in

H5V and BAE cells, respectively; $p < 0.005$ for both). This group showed improved adhesion also compared to each HIF α group (HIF-1 α + HIF-2 α /HIF-1 α : 1.48 ± 0.14 -fold and 1.66 ± 0.26 -fold in H5V and BAE cells, respectively; $p < 0.005$ for both; HIF-1 α + HIF-2 α /HIF-2 α : 1.38 ± 0.13 -fold, $p < 0.005$, and 1.09 ± 0.04 -fold, $p < 0.05$, in H5V and BAE cells, respectively).

Assessing the ability of ECs to adhere to U937 human monocytes (fig. 2b), we also found increased adherence following HIF α overexpression (HIF-1 α : 1.91 ± 0.47 -fold and 2.57 ± 0.48 -fold in H5V and BAE cells, respectively; $p < 0.05$ for both; HIF-2 α : 2.84 ± 0.37 -fold and 3.57 ± 0.65 -fold in H5V and BAE cells, respectively; $p < 0.005$ for both; HIF-1 α + HIF-2 α : 3.01 ± 0.54 -fold and 3.47 ± 0.25 -fold in H5V and BAE cells, respectively; $p < 0.005$ for both). These effects were significantly reduced in the presence of the HIFdn construct. Interestingly, HIF-2 α consistently appeared to have an added effect on the adhesive capacity of ECs compared to HIF-1 α .

Fig. 2. Adhesion capacity of ECs following HIFα overexpression. **a** Adhered cells were stained using XTT-based colorimetry and results are presented as OD values standardized to control. Shown are the results for adherence of HIFα-transfected H5V cells and BAE cells to fibronectin-coated plates. Cotransfection of HIFα subunits with HIFdn served to attenuate the effect of HIF-1/2α on adhesion. **b** U937 monocyte adherence to HIFα-transfected H5V cells and BAE cells was also studied. **c** Representative flow cytometry analysis of ICAM-1 and VCAM-1 cell surface expression in H5V cells following HIFα or empty vector transfection. Average mean fluorescence intensity (MFI) relative values $\pm$ SD of 3 independent assays are presented. * $p < 0.05$; ** $p < 0.005$.

Fig. 3. The effect of HIF α overexpression on EC paracrine effects. **a** Migration capacity of H5V cells and BAE cells following incubation in HIF α -overexpressing cell-conditioned media. **b** H5V cells were seeded on Matrigel-coated dishes and incubated in

HIF α -overexpressing cell-conditioned media. Tube formation scoring was performed as described in Materials and Methods. Results representative of 4 independent experiments are shown. * $p < 0.05$; ** $p < 0.005$.

Finally, we examined the surface expression of ICAM-1 and VCAM-1, previously shown as essential during angiogenesis [29, 30], following HIF α overexpression. Flow cytometry analysis indeed revealed elevated expression (1.7- to 2.9-fold; $p < 0.005$) of both ICAM-1 and VCAM-1 following HIF α transfections (fig. 2c).

Paracrine Effects of Conditioned Medium of ECs Overexpressing HIF-1 α and HIF-2 α

In order to address the contribution of HIF-1 α and HIF-2 α to the angiogenic paracrine profile of ECs, we tested the migratory capacity of wild-type H5V and BAE

cells towards the conditioned medium from ECs overexpressing HIF α subunits (fig. 3a). The conditioned media of both HIF-1 α - and HIF-2 α -overexpressing cells were found to induce migration (HIF-1 α : 2.29 ± 0.36 -fold and 2.46 ± 0.3 -fold in H5V and BAE cells, respectively; $p < 0.005$ for both; HIF-2 α : 1.43 ± 0.22 -fold, $p < 0.05$, and 2.33 ± 0.36 -fold, $p < 0.005$, in H5V and BAE cells, respectively) to a greater extent than control media. Co-transfection of HIFdn mutant together with each HIF α subunit reduced the number of invading cells. While in the H5V cell study, increased migration toward HIF-1 α -compared to HIF-2 α -derived medium was observed, this

Fig. 4. Antibody arrays of HIF α -expressing ECs. **a, b** H5V cells transfected with empty vector, HIF-1 α , HIF-2 α or HIF-1 α and HIF-2 α were analyzed for their cytokine expression. Each experimental group included 2 different array membranes containing duplicates of a total of 96 different spots of specific antibodies.

Signal quantification was performed using Tina 2.0 software, and the threshold indicative of altered protein expression (upregulated or downregulated) was set as 1.5-fold. The presented cytokines showed such modification in at least 1 group ($p < 0.05$).

trend failed to reach statistical significance in BAE cells. However, in both cell lines, the most prominent migration was measured toward the HIF-1 α and HIF-2 α -co-transfected cell medium (3.17 ± 0.34 -fold and 3.05 ± 0.24 -fold in H5V and BAE cells, respectively; $p < 0.005$ for both). Importantly, statistical significance was main-

tained when comparing this last group with either the HIF-1 α or HIF-2 α group (HIF-1 α + HIF-2 α /HIF-1 α : 1.41 ± 0.19 -fold and 1.23 ± 0.14 -fold in H5V and BAE cells, respectively; $p < 0.05$ for both; HIF-1 α + HIF-2 α /HIF-2 α : 2.26 ± 0.28 -fold, $p < 0.005$, and 1.31 ± 0.21 -fold, $p < 0.05$, in H5V and BAE cells, respectively).

Fig. 4. c Representative detection of signals of selected genes is also shown. cont-GFP = Green fluorescent protein.

We next explored the effect of conditioned medium from ECs overexpressing HIF- α on the ability of wild-type ECs to form capillary-like tubules on Matrigel-coated plates. Accelerated progression of the tubulogenic process was evident following exposure to HIF α -transfected cell media (fig. 3b). Correlating with the migration assay results, HIF-1 α -transfected cell medium promoted significantly more extensive tube formation compared to HIF-2 α . Finally, tube formation was more robust following incubation in conditioned medium obtained from cotransfected ECs, showing closed polygon structures as soon as 8 h after plating.

HIF-1 α and HIF-2 α Additively Influence the Expression of Multiple Angiogenic Proteins

To study the effect of HIF on the expression of a wide range of cytokines and receptors with major importance in the autocrine and paracrine response of ECs, cytokine

arrays were used. Comparative analysis of cell extracts of ECs overexpressing HIF-1 α and HIF-2 α revealed distinct cytokine expression patterns, which included multiple proteins not previously associated with HIF transcriptional activity (fig. 4a–c). SDF-1 α , GM-CSF, IL-10, IGFBP-3, TIMP-1, Fas ligand, osteopontin, AXL and Dtk as well as the receptors CXCL16, leptin R and P-selectin were induced in HIF-1 α -overexpressing cells, but not following HIF-2 α induction. Conversely, Flt3 ligand, VEGFR-2, IL-13, sTNFR-1 and DDPIV/CD26 were induced in the HIF-2 α but not in the HIF-1 α group.

Combined HIF-1 α and HIF-2 α overexpression was associated with an additive effect on the expression of a large number of multifunctional proteins. Several proteins classified as proangiogenic, including the factors VEGF, SCF, GM-CSF, MCP-1, MMP-2, IL-13 and TPO and the receptors VEGFR-1, leptin R, sTNFR-1 and P-selectin, were further upregulated following HIF-1 α and HIF-2 α cotransfection. On the other hand, antiangiogenic factors such as PF4, IFN γ , MIG and IL-2 were repressed in the coexpression group. Nevertheless, down-regulation of proangiogenic factors such as Shh-N and pro-MMP-9, as well as upregulation of antiangiogenic factors, such as TIMP-1 and IL-10, were also noticed.

HIF-1 α and HIF-2 α additively contribute to angiogenic vessel formation in vivo. Based upon the findings achieved in the in vitro studies, we aimed to assess the contribution of HIF-1 α and HIF-2 α to in vivo angiogenesis using a mouse Matrigel plug assay. Anti-CD31 staining of the excised plugs revealed increased vascular density following injection of HIF α -overexpressing ECs (fig. 5). A tube formation assay revealed that while HIF-1 α overexpression was associated with a more advanced vasculogenic process and larger vessel diameter, HIF-2 α contributed to vessel number, showing numerous relatively small, immature capillaries. The combination of HIF-1 α and HIF-2 α was characterized by a considerable increase in vascular density, as well as a further developed and robust tube formation process compared to other groups (HIF-1 α : 2.9 ± 0.43 -fold; HIF-2 α : 1.94 ± 0.36 -fold; HIF-1 α + HIF-2 α : 4.35 ± 0.51 -fold; $p < 0.005$).

Discussion

Several recent studies have reported the differential roles of HIF-1 α and HIF-2 α in various cell types [11–14]. The present study highlights the improved vasculogenic properties consequent to overexpression of HIF-1 α or

Fig. 5. The effect of HIF overexpression on vessel formation in vivo using a Matrigel angiogenesis assay. Four groups ($n = 5$) of C57/BL mice were subcutaneously injected with 2×10^6 H5V cells overexpressing HIF-1 α , HIF-2 α or both, mixed with liquid Matrigel. After 8 days, mice were euthanized and plugs were removed for vessel analysis. Sections from the center of the excised plugs were stained with CD31 and hematoxylin. Quantitative analysis of CD31-positive vascular density was performed under a light microscope ($\times 40$) in high-power fields ($n = 10$), employing Image J software. MVA = Mean vessel area; GFP = green fluorescent protein. ** $p < 0.005$.

HIF-2 α in ECs. Additionally, the pattern of HIF-1 α and HIF-2 α coexpression in ECs was investigated, in order to explore further biological resemblance with the functional response to angiogenesis-inductive conditions such as hypoxia. Collectively, our results point to a consistent common effect of HIF-1 α and HIF-2 α on vascular formation, which might be attributable to potential complementary roles during vessel formation.

The protein array data point to differential effects of HIF-1 α versus HIF-2 α on the expression of angiogenesis-related proteins in ECs. The modified expression of the cytokines described above might ensue both directly or indirectly from HIF α overexpression and thus remains to be specifically determined for each cytokine. One example, for an indirect pathway, might be the increased MCP-1 expression, potentially resulting from the VEGF-MCP-1-HIF-1 α induction loop [31]. However, several genes encoding proteins that showed elevated expression, such as VEGF, SDF-1 α , VEGFR-1 and VEGFR-2, are well-established HIF targets containing HIF-binding domains in their promoter/enhancer region [5, 7, 9, 10, 32]. The genes encoding additional observed proteins, such as leptin/TNF receptors, AXL and IGFBPs, were previously shown to be upregulated in ECs following HIF α stabilization [2]. Certain growth factors, such as MMP-2 and Fas ligand, were shown to be altered in ECs under hypoxic conditions [33, 34] but not directly associated with HIF.

These distinct transcription patterns may account in part for the diverse effects of HIF-1 α and HIF-2 α activity in ECs. Formerly, HIF-1 α was shown to regulate the expression of adhesion molecules on trophoblast stem cells and leukocytes [35, 36]. Induction of HIF-1 α in vascular smooth muscle cells was actually associated with decreased adhesion and migration [37]. Recently, constitutive expression of HIF α was demonstrated to promote EC migration and tube formation capacity [2, 10]. Our results suggest for the first time a contribution of HIF-1 α and, more prominently, HIF-2 α , to the adhesion capacity of ECs. Several studies have indeed suggested a dominance of HIF-2 α regulation over HIF-1 α in ECs [5, 7, 9, 10, 32]. Consistent with the enhanced cell-to-cell adhesion mediated by HIF α , we found increased cell surface expression of VCAM-1 and ICAM-1 in HIF α -overexpressing ECs. These 2 adhesion molecules have been shown to play important roles in EC-mediated proangiogenic properties [29, 30].

We have also shown here that HIF overexpression induced robust paracrine effects, promoting EC migration and tubular organization, in which HIF-1 α appeared particularly prominent. Whereas both HIF-1 and HIF-2 drove expression of VEGF, MCP-1 and SCF, other proteins, such as SDF-1 α and GM-CSF, were significantly induced solely following HIF-1 α overexpression. This may point towards complementary functions of HIF α subunits in angiogenic regulation. Indeed, both HIF isoforms were recently shown to be temporally and spatially activated during glomerulogenesis, pointing at the potential involvement of HIF-1 α in tubulogenesis and HIF-2 α in vasculogenesis [38].

One of the findings described in this study was the additive effect of HIF-1 α and HIF-2 α on EC activation. Co-expression of HIF-1 α and HIF-2 α induced a more robust effect on EC vasculogenic properties compared to those previously attributed to each one of the HIF α subunits expressed separately both in vitro and in vivo. Additionally, changes in the expression of various cytokines following overexpression of one of the HIF α subunits were further increased by the simultaneous expression of the other.

There are several potential explanations for the additive effects of the 2 HIF α subunits. Firstly, the common, saturable mechanism of VHL-mediated HIF α degradation is likely to contribute to a mutual positive interaction between HIF-1 α and HIF-2 α [14, 26, 27]. Interestingly, this positive interaction was demonstrated to be abolished in VHL-defective cells [27]. Additionally, HIF-2 α preferentially stimulates the expression of certain HIF-1 α angiogenic targets through binding to the HRE or other response elements [5, 8–10]. Such activity of HIF-2 α might increase the transcription of HIF α targets, when coexpressed with HIF-1 α , and thus widen the HIF α transcriptome to include other potential unique HIF-2 α targets. Finally, several reports have suggested

endogenous HIF α -specific inhibitors acting either at the mRNA or protein levels on one or both HIF α subunits [39–41]. Distinct tropism of inhibitors to each HIF α subunit might repress a single HIF α subunit, while the other still functions to some extent. Further investigation of the HIF-1 α and HIF-2 α interaction will be required to figure out the consequences of their common activity.

In conclusion, the present study further elucidates the differential effects of HIF-1 α and HIF-2 α on EC protein expression and vasculogenic properties. Moreover, while the exact mechanism is yet to be determined, we claim for the first time an additive effect of HIF-1 α and HIF-2 α on angiogenesis. These findings may pave the way for a better understanding of the endothelial physiological response to hypoxic conditions, leading to more efficient genetic-based clinical applications.

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